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공학석사학위논문

미세조류 *Chlorella vulgaris* 의 성장 향상을 위한
광전환 형광 염료 합성 및 특성 분석

Synthesis and characterization of fluorescent dyes and their
applications for the enhancement of growth rate of
Chlorella vulgaris

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서울대학교 대학원

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ABSTRACT

Four fluorescent dyes were carefully designed to convert wavelengths of light specifically favourable for microalgal growth in which a portion of white light was converted from green (500 – 580nm) to orange-red (580 – 650nm). Synthesized dyes were applied to microalgae cultivation system by two methodologies: (1) light-converting layer, and (2) light-converting medium in which the dyes were dissolved in either ethanol or water to form light-converting layer between microalgae cultures and the light source. Under converted light provided by both methods, *Chlorella vulgaris* cultures showed maximum increase in lipid productivity by 13.00% and 17.15%, and biomass productivity by 31.65% and 37.66% compared to the control. Overall, the cultivation results confirmed beneficial effects of modified light on microalgae cultures both on their lipid and biomass productivities. Note that both growth parameters achieved higher performance in the light-converting medium thereby the effectiveness of the cultivation method was validated.

Keywords: organic dyes, perylene, light-conversion, wavelength, growth, microalgae, cultivation

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CHAPTER I: Introduction

Microalgae is considered a promising energy source over depleting fossil fuels for its renewable, biodegradable, and environmentally-friendly properties. Cultivation of microalgae received attention mainly to increase lipid content such as triacylglycerol known as feedstock for biofuel production. In addition, many studies were carried out to optimize various cultivation conditions including light, temperature, gas transfer, mixing technique, and pH. In this particular study, light-related parameters such as light intensity, illumination time, and light source were controlled to develop a new strategy for microalgae cultivation.

Light typically acts as a driving force in photoautotrophic cultivation. Photons are absorbed by microalgae cells to stimulate photosynthesis thus the quality of light in terms of intensity and wavelength is critical for cell growth¹. Algal photosynthesis occurs most efficiently in photosynthetically active radiation (PAR) in which some microalgae strains grow faster under certain colours, particularly in blue (400 – 500 nm) or red (600 – 700 nm) regions. However, these regions account for less than 15% of total solar radiation. Theoretically, if less useful wavelength regions were made available for photosynthetic activity, the overall productivity of biomass and lipid would be greatly improved². It would also make algal cultivation possible in diverse locations, including those with suboptimal light.

To enable efficient utilization of solar radiation, various fluorescent dyes were synthesized to absorb light in green (500 – 580 nm) region and emit light in a bathochromically shifted region. These light-converting dyes were applied either in separate layers adjacent to microalgae cultures or directly dissolved in the culture medium during cultivation. Both methodologies utilized dyes in solution form to secure higher quantum yield, lower cost, and less toxicity over solid form in which fluorescence of dyes can be severely hindered especially when coated on polymethyl methacrylate (PMMA) filter³.

Light-converting medium, which is a mixture of a light-converting dye and culture medium adds multifunctionality to a common medium. The proposed method is simple, space-efficient, and economically feasible. No additional layers are needed for light conversion, nor requires larger area to build more layers. Despite environmental concerns on coloured water, the recyclability of medium prevents drainage into open water, and when necessary, easy separation of dyes is also possible using self-assembly-induced mechanisms.

The efficacy of both methodologies was evaluated in terms of lipid and biomass productivities of microalgae while all other aspects of the experiment were designed to mimic optimal cultivation conditions⁴⁻⁶. Simultaneously, the effect of different colours on the growth of microalgae is also discussed.

CHAPTER II: Background and Literature Review

1. The rise of 3rd generation biomass, microalgae, as an alternative energy source

Global energy demand is constantly increasing yet the fossil fuel depletion still remains a severe environmental issue around the world. Extensive studies have suggested alternative energy sources including solar, wind, hydro, geo-thermal, though, most have failed to reach commercialization primarily due to topographic limitations and economic feasibility. In this context, renewable and environmentally-friendly biomass energy can be regarded as one of the most appropriate solution, since biomass is the only energy-containing carbon resource that is large enough in quantity to be used as a substitute for fossil fuels⁷. Bioenergy was researched all around the world starting from the 1st generation through to 3rd generation biomass, microalgae, which offer numerous advantages over preceding generations.

1st generation biomass such as corn and sugarcane merely offers any advantage over fossil fuels with regards to greenhouse gas emission. Instead, it arouses controversy in research ethics as the ingredients are also food sources. The corresponding ingredients skyrocketed in prices and aroused concerns on competition for food. 1st generation biomass soon lost popularity.

2nd generation biomass uses lignocellulosic plants and agricultural residues that generate higher energy yields compared to corn and sugarcane. Also, these new energy sources address many issues associated with the 1st generation biomass, majorly regarding competition for food crops and arable

land usage. However, entire energy generation process is more complex, and hydrolysis of cellulosic biomass into sugars still remains an unresolved problem in the pretreatment step.

On the contrary, microalgae suffer no such obstacles. Instead, microalgae can produce abundant lipids which can be extracted from cells and converted into methyl or ethyl esters of fatty acids via transesterification to produce biofuel. The biomass can also reuse and recover waste nutrients and greenhouse gas utilizing non-productive land and resources. Consequently, these all contributed to the emergence of microalgae as one of the most valuable energy source.

2. Previous work on microalgae engineering

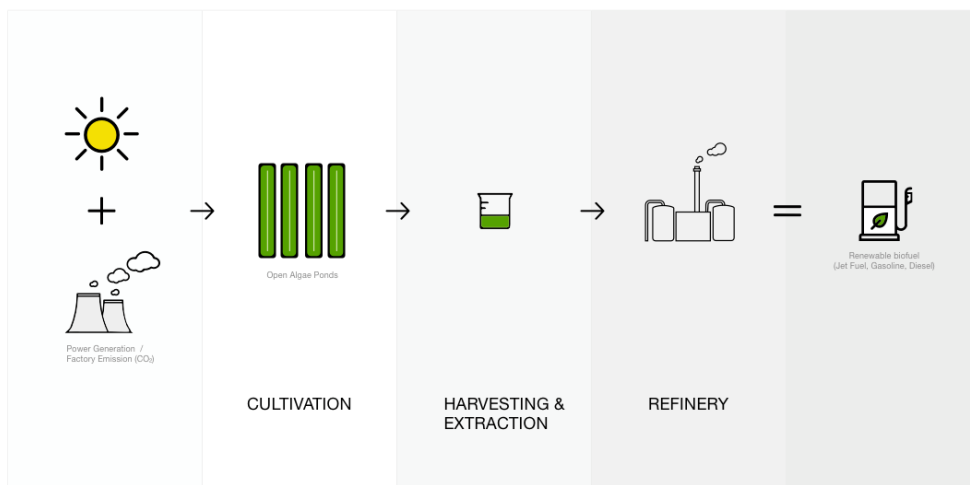
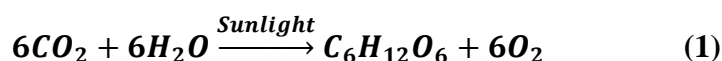


Figure 1 Biodiesel production line using microalgae as an energy source (FunkJeffrey, 2015)

Previous studies focused on every aspect of biodiesel production (**Fig. 1**) in order to boost growth, produce desired products in excessive amount, and to accelerate reproduction rate. Various microalgae species were genetically engineered to bear rich content of growth determining constituents including photosynthetic pigments, fatty acids, and other high value products⁸. Many outdoor cultivations investigated the effect of temperature, mixing technique, gas transfer control, and pH changes in bioreactors on biomass growth^{3,9–12}. Furthermore, new methods for efficient extraction and refinery have been intensely scrutinized. In this particular study, the focus was on cultivation where the beneficial effect of various light conditions on microalgae growth was tested.

3. Microalgae cultivation and the light source

A challenge was to optimize light conditions to positively affect cell growth. Since microalgae photosynthesize to produce carbon derivatives for growth and lipid production, it makes logical sense to provide utmost light energy (**Eq.1**).



However, microalgae are unable to make full use of direct sunlight which is an inexhaustible source of energy consisting of all colours of the spectrum. Appropriate amount of light corresponding to absorption capacity sensibly matches an effective light condition for microalgae.

Also, microalgae can utilize a certain fraction of photosynthetically active radiation (PAR) more efficiently than others (**Fig. 2**). This is primarily due to the photosynthetic pigments in the cells, chlorophyll a and chlorophyll b, that absorb orange-red (600 – 700 nm) and reflect off green light. Inferred from spectral properties of chlorophyll a and chlorophyll b, providing orange-red light would significantly affect the growth of microalgae.

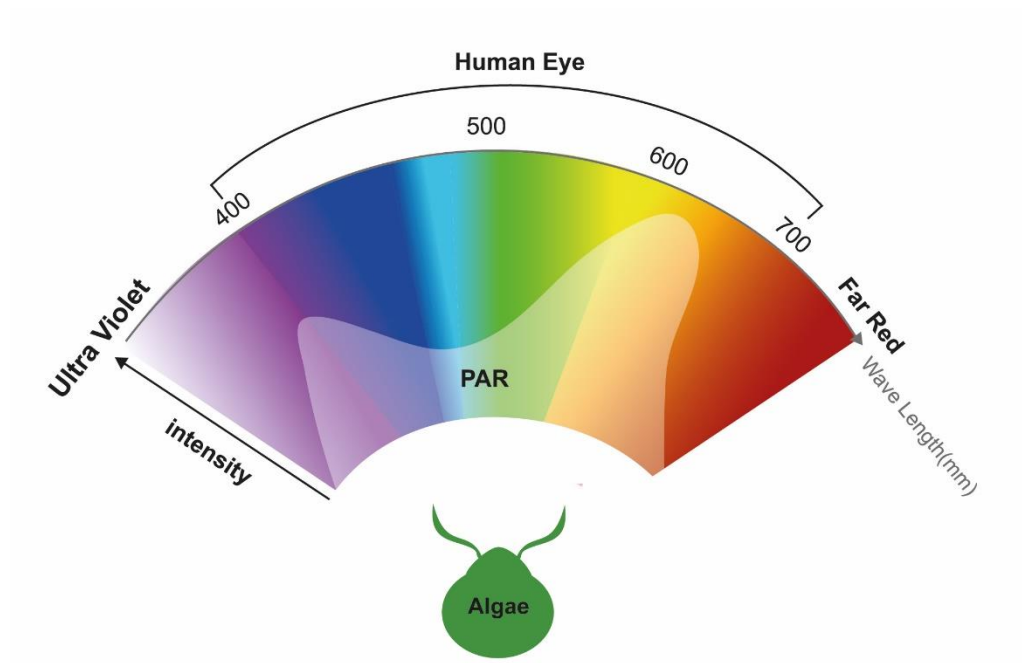


Figure 2 Photosynthetically active radiation of chlorophyll a- and chlorophyll b-containing organisms

In previous studies, Mohesenpour et al.³ tested the effect of red light on microalgae growth and reported 20 – 70% increase in dry weight. Wondraczek et al.¹³ also carried out a similar cultivation using fluorescence phosphorus powder derived from Eu^{2+} as a light converter and obtained 35% increase in cell weight. These promising results assured practicability of using red-emitting dyes in microalgae cultivation.

4. Design and synthesis of light-converting dyes

Light-converting dyes offer a potential solution to the proposed goal as they can manipulate spectral properties of the light source to provide more energy in the photosynthetically active region (PAR). Four fluorescent dyes were synthesized for this purpose to absorb light in the green wavelength range (500 – 580 nm) and emit in the orange-to-red wavelength range (580 – 650 nm). Their emission spectra differed by small increments on a nanometer scale so various shade of orange and red lights were tested.

Perylene moiety was selected in this study for its relatively high quantum yield, high thermal and chemical stability which satisfy essential requirements for later application during cultivation. Perylene also allowed structural modifications fairly straightforward by attaching substituents at peripheral and bay positions to tweak chemical and physical properties. Additionally, the chemicals used in the synthesis process were relatively cheap and less toxic compared to reported studies¹³.

Structure of synthesized dyes are shown in **Fig. 3**.

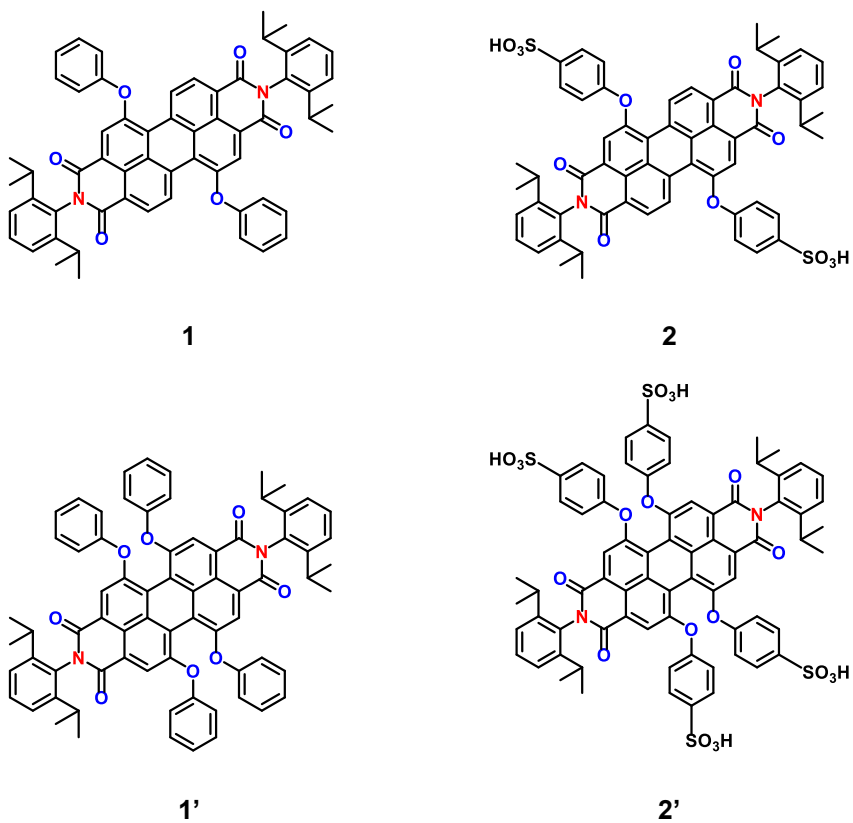


Figure 3 Structure of synthesized dyes

5. Bioreactor design and cultivation methodologies

Fluorescent dyes were applied in cultivation system by two different methodologies to create light-converting effect. Dyes **1** and **2** were dissolved in ethanol in individual flasks and were installed between light source and each microalgal cultures. Dyes **1'** and **2'** were dissolved in culture medium to act as a coloured medium. Since microalgae cultures were directly exposed to the medium, toxicity and concentration of dyes was carefully adjusted through preliminary tests. Microalgae cultures grown either in light-converting layer or light-converting medium systems were later compared on growth rate.

Medium selection was rather important as it signifies a living environment for microalgae to grow in. TAP medium was first considered as it would stimulate fast growth of microalgae. However, the TAP medium was ruled out because it contains carbon source in the form of acetic acid. Microalgae should only grow on carbon products formed via photosynthetic processes to test the effect of modified light. Alternative choice was BG-11 medium. No excessive nutrients were supplied and the cells must photosynthesize to produce carbon source either to replicate or accumulate lipid in their bodies.

Autotrophic environment was sustained throughout the experiment.

6. Species selection

Chlorella vulgaris species was selected as a local representative of eukaryotic microalgae. They are also known to show increased growth rate under red light^{2,13}. *C. vulgaris* contains lots of lipid, and enclose chlorophyll a and chlorophyll b in their cell bodies to absorb light in 400 – 500 nm and 600 – 700 nm ranges to photosynthesize as shown in **Fig. 4**¹⁴. Main focus was on the red shade of light, and experiments were planned to examine if *C. vulgaris* grew more efficiently when extra light was provided in this particular wavelength range.

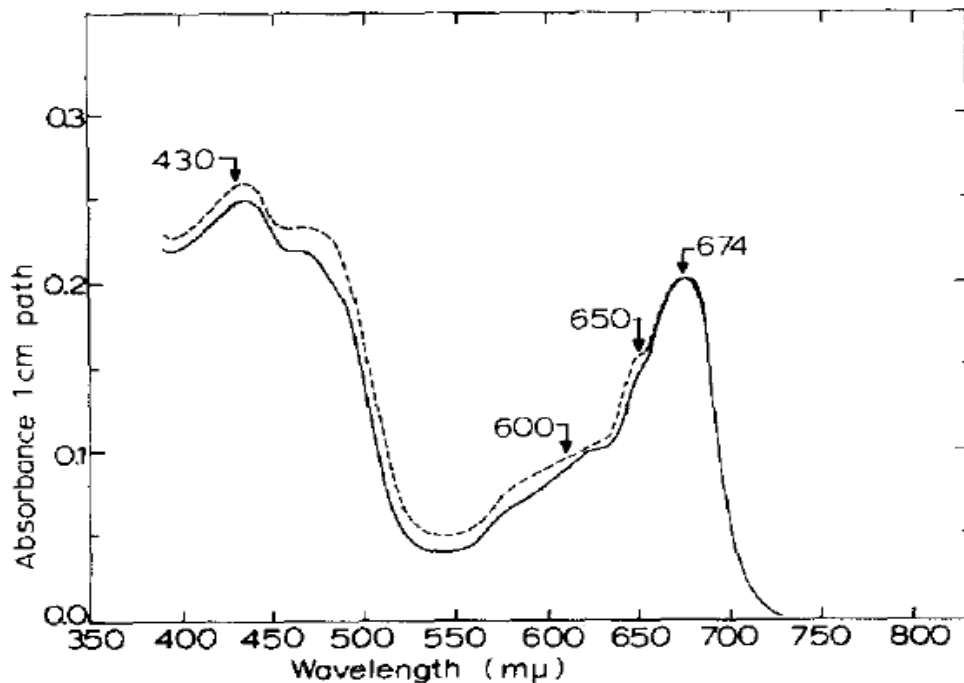


Figure 4 Absorption spectrum of *Chlorella vulgaris*¹⁴

CHAPTER III: Methods – Synthesis

1. *Synthesis*

1.1 Chemicals

Perylene-3,4,9,10-tetracarboxylic dianhydride, 2,6-diisopropylaniline, propionic acid, sulfuric acid, bromine, acetic acid, N-Methyl-2-pyrrolidone (NMP), anhydrous potassium carbonate, and phenol purchased from Sigma-Aldrich, and dichloromethane purchased from TCI Chemicals were used. Other reagents and solvents were obtained from various commercial suppliers including Alfa Aesar, Duksan Chemicals, and Samchun Chemicals at reagent-grade quality.

1.2 Synthesis of 1,7-dibromo-perylene-3,4,9,10-tetracarboxydiimide

Perylene-3,4,9,10-tetracarboxylic dianhydride (39.2 g, 0.10 mmol), iodine (0.474 g, 3.73 mmol), and 450 mL of 98% sulfuric acid were mixed and stirred for 2 hours at room temperature. The reaction temperature was increased to 80°C and bromine (8.87 mL, 172 mmol) was added dropwisely. The mixture was heated for 16 hours then cooled to room temperature while displacing excess Br₂(g) by flowing N₂(g) into the reaction flask. The mixture was slowly transferred to a litre of ice-water and red precipitate was obtained. The precipitate was collected by suction filtration and was washed with distilled water several times until the aqueous layer became neutral to yield crude product. The product was dried in a vacuum oven at 80°C (Yield: 94%).

1.3 Synthesis of N,N'-Bis(2,6-diisopropylphenyl)-1,7-dibromoperylene-3,4,9,10-tetracarboxydiimide

1,7-dibromoperylene-3,4,9,10-tetracarboxydiimide (5.50 g, 0.01 mol) was suspended in 320 mL of propionic acid in a 500 mL round-bottom flask at 70°C for 1 hour. 2,6-diisopropylaniline (13.2 mL, 0.07 mol) was added to the mixture after increasing the reaction temperature to 140°C and heated overnight. The mixture cooled to room temperature and was slowly transferred to 500 mL of methanol. Filtrate was collected after suction filtration; washed thoroughly with methanol. Crude product was obtained after evaporating the filtrate in rotatory evaporator. The product was purified by column chromatography on silica gel using CH₂Cl₂ as the eluent. A band containing tribrominated diimide was separated first, followed by dibrominated and monobrominated (yield: 53%).

1.4 Synthesis of N,N'-Bis(2,6-diisopropylphenyl)-1,7-diphenoxy-perylene-3,4,9,10-tetracarboxydiimide (1)

N,N'-Bis(2,6-diisopropylphenyl)-1,7-dibromoperylene-3,4,9,10-tetracarboxydiimide (4.0 g, 4.62 mmol) was suspended in 280 mL of N-Methyl-2-pyrrolidone (NMP) with phenol (0.94 g, 10.0 mmol), and anhydrous K₂CO₃ (2.8 g, 20.3 mmol). The mixture was heated at 120°C for 24 hours then cooled to room temperature. The mixture was slowly poured into 300 mL of 5% HCl solution. A precipitate was then collected by suction filtration, repeatedly washed with distilled water. Crude product was dried in vacuum oven at 80°C overnight. The product was purified by column chromatography on silica gel using CH₂Cl₂ as the eluent.

Yield: 82.8%; MALDI-TOF MS: m/z 896.25, 100% [M + H⁺].

1.5 Synthesis of N,N'-Bis(2,6-diisopropylphenyl)-1,7-di-[4-(sulfuric acid)phenoxy]perylene-3,4,9,10-tetracarboxydiimide (2)

N,N'-Bis(2,6-diisopropylphenyl)-1,7-diphenoxy-perylene-3,4,9,10-tetracarboxydiimide (1 g, 1.12 mmol) was added to 2.5 mL of concentrated sulfuric acid and the flask was sealed. The mixture was stirred at room temperature for 15 hours. 5 mL of distilled water was added dropwisely to form a precipitate, then filtered under low pressure. The solid product was washed several times with 30 mL of CH₂Cl₂. Red product was dried in a vacuum oven at 80°C.

Yield: 75%; MALDI-TOF MS: m/z 1057.15, 100% [M + H⁺].

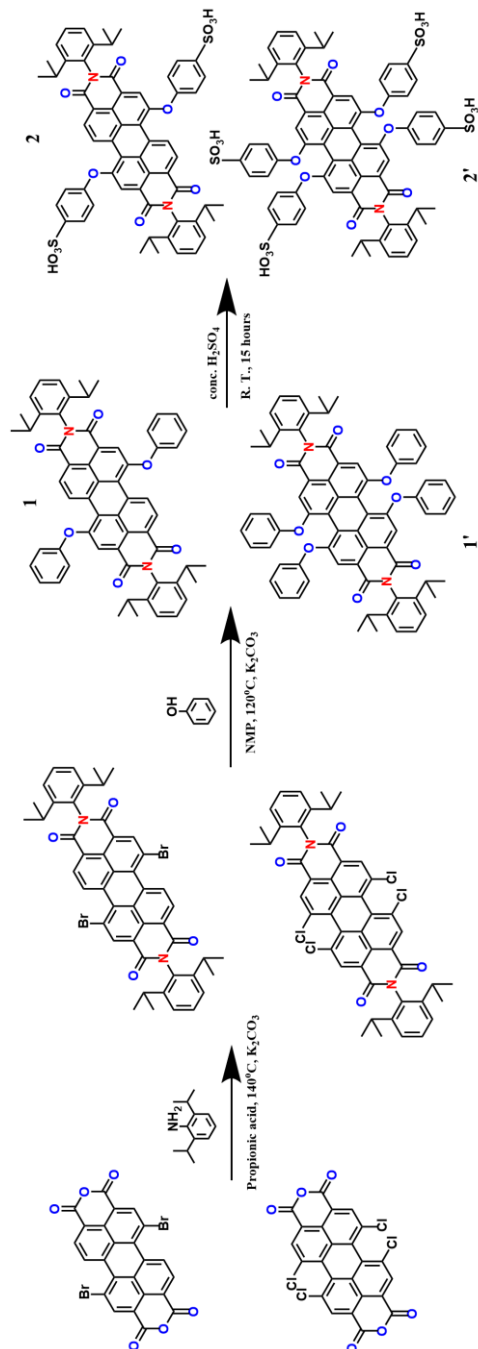


Figure 5 Synthetic scheme of dyes 1, 2, 1', and 2'

Structurally similar dyes were also synthesized using same substituents, this time tetra-substituted at bay positions instead of di-substitution. These dyes **1'** and **2'** were designed to show bathochromically shifted absorption and emission spectra compared to those of **1** and **2**. Theoretically, tetra-substituted dyes should provide redshifted light closer toward the targeted wavelength range.

1.6 Synthesis of 1,6,7,12-tetrachloroperylene-3,4,9,10-tetracarboxydiimide

Perylene-3,4,9,10-tetracarboxylic dianhydride (39.2 g, 0.10 mmol), iodine (0.474 g, 3.73 mmol), and 450 mL of 98% sulfuric acid were mixed and stirred for 2 hours at room temperature. The reaction temperature was increased to 80°C and chlorine (7.59 mL, 172 mmol) was added dropwisely. The mixture was heated for 16 hours then cooled to room temperature while displacing excess Cl₂(g) by flowing N₂(g) into the reaction flask. The mixture was slowly transferred to a litre of ice-water and red precipitate was obtained. The precipitate was collected by suction filtration and was washed with distilled water several times until the aqueous layer became neutral to yield crude product. The product was dried in a vacuum oven at 80°C (Yield: 93%).

1.7 Synthesis of N,N'-Bis(2,6-diisopropylphenyl)-1,6,7,12-tetrachloroperylene-3,4,9,10-tetracarboxydiimide

1,6,7,12-tetrachloroperylene-3,4,9,10-tetracarboxydiimide (5.30 g, 0.01 mol) was suspended in 320 mL of propionic acid in a 500 mL round-bottom flask at 70°C for 1 hour. 2,6-diisopropylaniline (13.2 mL, 0.07 mol) was added to the mixture after increasing the reaction temperature to 140°C and heated overnight. The mixture was cooled to room temperature and was slowly transferred to 500 mL of methanol. Filtrate was collected after suction filtration; washed thoroughly with methanol. Crude product was obtained after evaporating the filtrate in rotatory evaporator. The product was purified by column chromatography on silica gel using CH₂Cl₂ as the eluent. A band containing product was separated first, followed by tri-chlorinated, di-chlorinated and then mono-chlorinated (Yield: 76%).

1.8 Synthesis of N,N'-Bis(2,6-diisopropylphenyl)-1,6,7,12-tetraphenoxy-perylene-3,4,9,10-tetracarboxydiimide (1')

N,N'-Bis(2,6-diisopropylphenyl)-1,6,7,12-tetrachloroperylene-3,4,9,10-tetracarboxydiimide (3.91 g, 4.62 mmol) was suspended in 280 mL of N-Methyl-2-pyrrolidone (NMP) with phenol (0.94 g, 10.0 mmol), and anhydrous K₂CO₃ (2.8 g, 20.3 mmol). The mixture was heated at 120°C for 24 hours then cooled to room temperature. The mixture was slowly poured into 300 mL of 5% HCl solution. A precipitate was then collected by suction filtration, repeatedly washed with distilled water. Crude product was dried in vacuum oven at 80°C overnight. The product was purified by column chromatography on silica gel using CH₂Cl₂ as the eluent.

Yield: 86%; MALDI-TOF MS: m/z 1080.29, 100% [M + H⁺].

1.9 Synthesis of N,N'-Bis(2,6-diisopropylphenyl)-1,6,7,12-tetra-[4-(sulfuric acid)phenoxy]perylene-3,4,9,10-tetracarboxydiimide (2')

N,N'-Bis(2,6-diisopropylphenyl)-1,6,7,12-tetraphenoxy-perylene-3,4,9,10-tetracarboxydiimide (1.21 g, 1.12 mmol) was added to 2.5 mL of concentrated sulfuric acid and the flask was sealed. The mixture was stirred at room temperature for 15 hours. 5 mL of distilled water was added dropwisely to form a precipitate, then filtrated under low pressure. The solid product was washed several times with 30 mL of CH₂Cl₂. Red product was dried in a vacuum oven at 80°C.

Yield: 84%; MALDI-TOF MS: m/z 1401.06, 100% [M + H⁺].

1.10 Characterization

Synthesized dyes were examined for chemical analysis via matrix-assisted laser desorption / ionization time of flight (MALDI-TOF) mass spectrometry. MALDI-TOF MS was measured on Voyager-DE STR Biospectrometry Workstation using α -cyano-4-hydroxy-cynamic acid (CHCA) matrix and substantiated molecular weight of dyes **1**, **1'**, **2**, and **2'**.

Spectral characteristics were analyzed using PerkinElmer UV/Vis spectrometer Lambda 25 and PerkinElmer fluorescence spectrometer LS 55, and absorption and emission spectra were scanned in 400 nm – 800 nm range. Quantum yield of dyes were measured using Jasco International FP-8500ST spectrofluorometer.

2. Cultivation

2.1 Strain and seed culture

Chlorella species are one of the most common microalgae cultured for biofuel production and widely known for its high productivity of fatty acids. They have relatively short and regular life cycle and are relatively easy to culture in a lab environment. Most importantly, cells absorb red light efficiently owing to their pigment content. Thereby, *Chlorella vulgaris* was selected as the working organism. *C. vulgaris* obtained from Algal Biomass Centre, Korea Advanced Institute of Science and Technology (KAIST) was cultivated in a sterilized BG-11 medium¹⁵ including NaNO_3 (1.5 g/L), K_2HPO_4 (0.058 g/L), Na_2CO_3 (0.020 g/L), and other essential components.

2.2 Optimization of photoautotrophic conditions in batch operation

Apart from miscellaneous lighting conditions, cells were cultivated in optimal conditions for photosynthesis^{4,16}.

When seeding, *C. vulgaris* was transferred from agar plate and inoculated in 250 mL culture flasks containing 200 mL culture medium. In order to prevent contamination during later stages of growth, the medium and flasks were sterilized in an autoclave for 20 minutes at 121°C beforehand. Cells were incubated at 24°C under cool white LED lamp which consistently illuminated at an intensity of 150 $\mu\text{mol}/\text{m}^2/\text{s}$. Flasks were clipped onto a shaker set at 120 rpm, and CO₂ enriched air was bubbled into mixture through silicon tubing via a 20 μm filter. The aeration was monitored using Dwyer's flowmetre, set to flow CO₂ enriched air (1%, v/v) at an air flow rate of 1.5 mL/min into the working volume. Gas input also contributed in mixing.

Inoculants were cultivated in clean condition starting from lag phase until the midst of exponential phase which lasted approximately 96 hours. Determined by optical density (OD) at 660 nm, the inoculants were transferred to a larger cultivation flask (500 mL with vented cap) for full-scale testing. A volume equivalent to 10% of the production medium was centrifuged at 3,000 rpm for 15 minutes to harvest cells. Then, the cell mass was washed twice with distilled water and re-suspended in 400 mL of BG-11 medium. Initial optical density of

fresh microalgae cultures was 0.1 when measured at 660 nm. Cells were grown until late-exponential phase where growth curve reached a plateau.

2.3 Application of light-converting dyes in microalgae cultivation

Dyes were added to the microalgae cultivation system by two different methodologies (**Fig.6**). The dyes were sorted according to their solubility in various solvents.

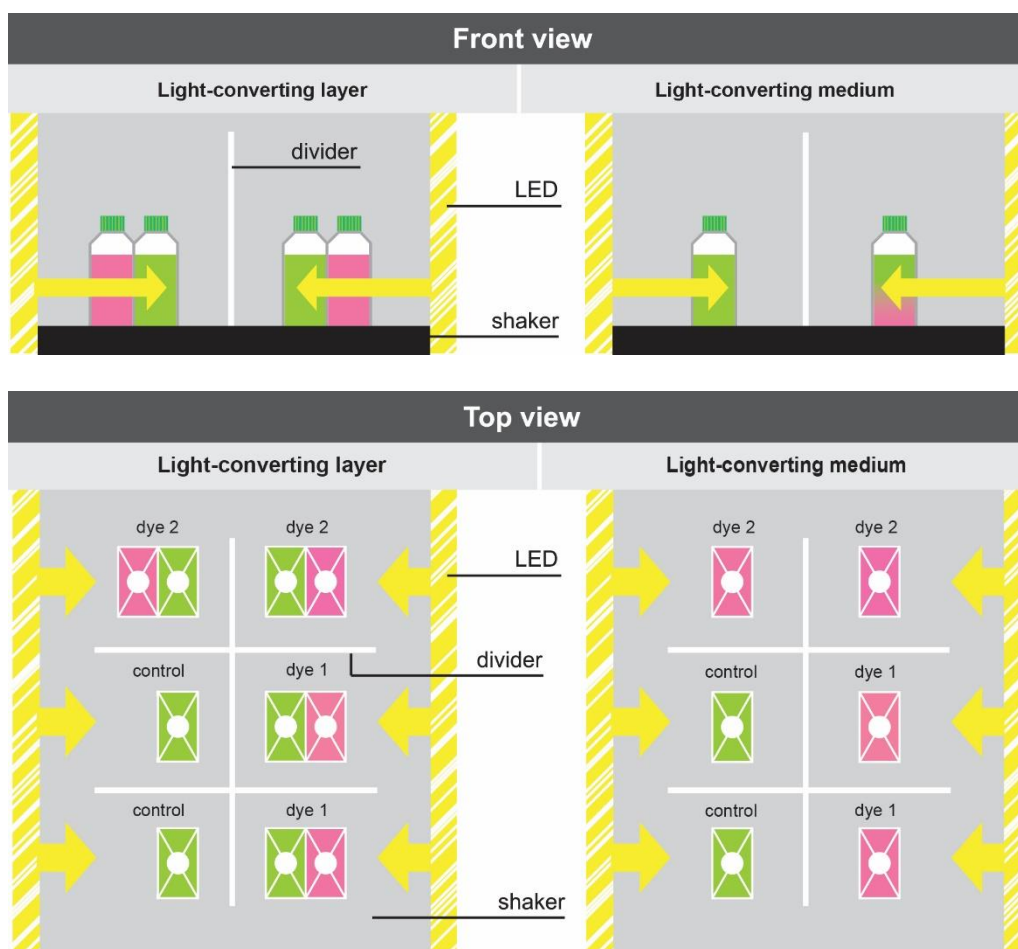


Figure 7 Front and top views of experimental setup for two cultivation methodologies: (left) dyes 1 and 2 dissolved in ethanol-base light-converting layer, and (right) dyes 1' and 2' dissolved in light-converting medium

2.3.1 Methodology 1: Light-converting layer

Organic solvent-soluble dyes **1** and **2** were each dissolved at 10^{-6} M in individual flasks containing 400 mL of ethanol only and microalgae were grown in separate flasks. Dye-containing flasks, acting as light-converting layers, were stacked against each microalgae flasks. The coupled flasks were placed next to the light source so that light had to penetrate through the dye solution prior to reaching the microalgae layer. The meniscus level in adjacent flasks were aligned to ensure the dye solution completely covered the culture flask surface. This way, the cells were not exposed to unmodified light shone directly from the source and only received modified light through the dye-containing flask. The dye-containing flasks were tightly sealed to prevent evaporation and change in dye concentration throughout cultivation.

As arranged in seeding step, the flasks were placed on Vision Scientific Co. LTD shaker set at 120 rpm to mix content thoroughly without touching the vented caps to avoid contamination. Carbon dioxide was supplied at the same rate as seed culture via silicon tubing (Korea Ace Scientific) for gas supply and mixing.

2.3.2 Methodology 2: Light-converting medium

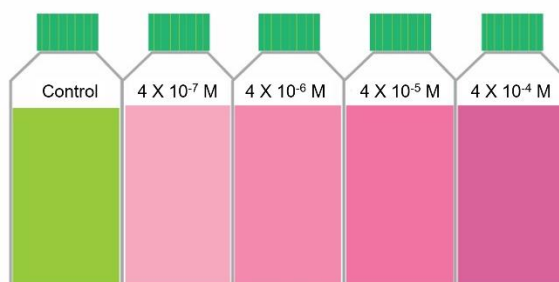


Figure 8 (Top) toxicity test of applying light-converting medium in microalgae cultivation, and (bottom) water-soluble dyes 1' and 2' tested at various concentrations

Prior to full-scale cultivation, a simple toxicity test was carried out to ensure microalgae can survive in the dye-medium complex (**Fig. 7**). Water-soluble dyes **1'** and **2'** were added at increasing concentration of 4×10^{-7} M, 4×10^{-6} M, 4×10^{-5} M, and 4×10^{-4} M to 100 mL of cell-containing BG-11 medium for testing.

Later, culture flasks containing BG-11 medium, 10^{-6} M of dyes **1'** and **2'**, and cells were tightly sealed and placed on Vision Scientific Co. LTD shaker set at 120rpm. The mixture was to mixed thoroughly without touching the vented caps to avoid contamination. Gas was supplied in the same manner for carbon supply and mixing.

2.4 Scheme of experiments

Both methodologies explained above followed same cultivation and sampling protocols after initial setup. Cells were cultured in a light room at 24°C for 120 hours under cool white LED lighting with a photon flux of approximately 100 $\mu\text{mol}/\text{m}^2/\text{s}$, an appropriate strength estimated to refrain from photolysis of dyes and photo-inhibition of cells. These conditions were consistently maintained and checked on a 12-hour basis throughout 120-hour cultivation.

Cell density was measured using Nexcelom Bioscience's Cellometer K2 Image Cytometer and Leica's optical microscope. Shimidaz Co's UV-Vis spectrophotometer was used to measure optical density. Dry cell weight (DCW) was also measured for 2nd methodology cultures to affirm cell density. For DCW measurement, a 5 mL sample was collected and fed through Whatman's cellulose nitrate membrane filter with 0.45 μm pore size. Quantum yield and lipid content were measured post harvesting, and photosynthetic activity and lipid productivity were calculated from the raw data obtained. All culture samples were experimented in duplicates, and sampled every 12 hours in triplicates.

3. Growth analysis

3.1 Cell density and biomass productivity

Cell density was determined from data obtained by Nexcelom Bioscience's Cellometer K2 Image Cytometer and Leica's optical microscope. Each slide had 20 µL of cells mounted on it for cell counting, and the cellometer counted cells individually to deduce cell density.

Cell density was plotted on a graph over time to show a growth curve that eventually levels off. Biomass productivity was calculated as shown (Eq.2)¹¹:

$$P_X = \Delta X / \Delta t \quad (2)$$

where P_X is the biomass productivity (g/d), ΔX is the change in cell density (cells/L).

3.2 Lipid content and lipid productivity

Once cells reached stationary growth phase, a small portion was used for quantum yield measurement, and the remaining for lipid extraction. It was performed gravimetrically based on a method adapted from Bligh and Dyer¹⁷. Then lipid content was calculated as per the following equation (**Eq.3**)¹²:

$$C_{Lipid} = \frac{\text{weight of extracted lipid}}{\text{weight of dried biomass}} \times 100 \quad (3)$$

Lipid productivity is a factor related to lipid content and biomass productivity. It was calculated as follows (Eq.4):

$$P_{Lipid} = \frac{C_{Lipid} \times DCW}{t} \quad (4)$$

where P_{Lipid} is lipid productivity (g/L·d), C_{Lipid} is lipid content (g/g), DCW is dry cell weight (g/L), and t is the cultivation period in days (d).

Sartorius balance was used to precisely measure the weight of dry biomass and extracted lipid to four decimal places.

3.3 Photosynthetic rate

Quantum yield of cells were measured once at the end of 120-hour cultivation. A small portion of cells were depleted of light for 30 minutes prior to testing in an Aquapen-C AP-C 100 Photon System Instrument. Blue and red excitation light at 455 nm and 620 nm were emitted at the intensity of up to 3,000 $\mu\text{mol}/\text{m}^2/\text{s}$ to estimate photon absorption of microalgae suspension. The amount of photon absorbed with respect to light emitted computes to quantum yield of cells which is pertinent to their photosynthetic rate.

CHAPTER IV: RESULTS AND DISCUSSION

Part 1: Synthesis

Dyes were designed to absorb and emit in specific wavelength ranges to provide extra light in need for microalgal growth.

Intermediates differ in structure only at the bay positions where one has two bromine atoms attached to it, and the other has four chlorine atoms. 2,6-diisopropylaniline was attached at both peripheral positions of the halogenated perylene, and these substituents increased the solubility in organic solvents. Next, phenols either di-substituted or tetra-substituted halogen atoms attached at bay positions. The benzene ring on phenols extended the pi-conjugation of electrons from the perylene body. This had an effect on spectral properties where the absorbance and emission peaks bathochromically shifted up to approximately 20 nm. Dyes **1** and **2** each showed maximum absorbance at 540.0 nm and 574.0 nm, and maximum emission at 583.0 nm and 613.5 nm, respectively.

Dyes **1** and **2** were each divided into two aliquot parts – one for use in light-converting layer, and another to proceed onto further synthetic reactions. Dyes **1'** and **2'** derived from dyes **1** and **2** via sulfonation under mild conditions. They showed maximum absorbance at 550.0 nm and 564.0 nm, and maximum photoluminescence at 599.0 nm and 619.5 nm. Note that the maximum absorbance of dyes **1'** and **2'** fall between those of dyes **1** and **2**. However, maximum emission of dyes **1'** and **2'** both exceed those of dyes **1** and **2**.

All dyes showed spectral characteristics as predicted in the design process. They absorbed most in the green wavelength region (mid-500 nm) in which chlorophyll-containing organisms would mostly reflect off. In return, they emitted in orange and red wavelength range (from late 500 nm to mid-600 nm) which roughly coincides with photosynthetically active region for microalgae.

Spectral characteristics of the light-converting dyes are presented in **Fig.**

8.

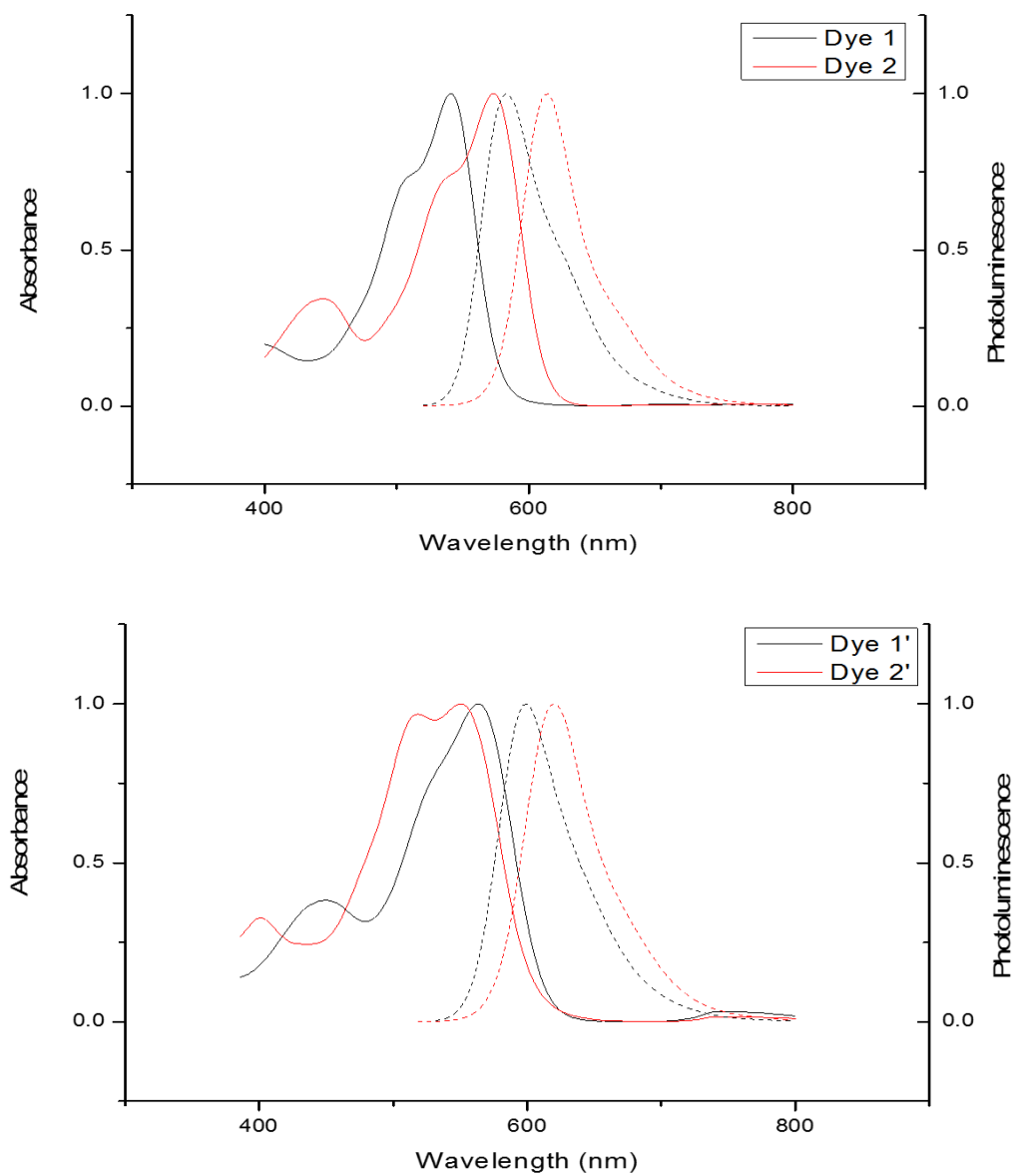


Figure 9 Normalized absorption and emission spectra of synthesized dyes

Table 1 Absorption and emission maxima, stoke shift, and quantum yield of dyes

Dye	Solvent	λ_{abs} (nm)	λ_{emi} (nm)	Stoke shift (nm)	Quantum yield
1	Ethanol	540.0	583.0	43.0	0.66
2		574.0	613.5	39.5	0.69
1'	Water	550.0	599.0	49.0	0.29
2'		564.0	619.5	55.5	0.35

Stoke shift for dyes **1**, **2**, **1'**, and **2'** were 43 nm, 39.5 nm, 49 nm, and 55.5 nm, respectively. Each pair of dyes showed similar Stokes shifts, however, the sulfonated dyes **1'** and **2'** have significantly broader spectra. Evidently, their average absorbance falls between the phenol-substituted dyes, and both their photoluminescence peaks exceed those of dyes **1** and **2**.

The emission spectra were almost evenly spread across orange to red. While interpretation of colours seem apparent, understanding the chemical mechanism behind the colour deviation is rather complicated. The complexity is due to the variety of inter- and intra-molecular interactions that cause spectral shifts. At the simplest level, solvatochromism explains that emission from fluorophores is hugely dependent on solvent polarity. As explained by Jablonski diagram (**Fig. 9**), a fluorophore is typically excited to the first singlet state (S_1) by energy intake. Energy level shifts up and down via internal conversion and vibrational relaxation, but returns to excited state μ_E almost instantly within 10 – 100 ps. Intersystem crossing is strongly dependent on solvent polarity where polar solvent molecules take part in the fluorescence mechanism by reorienting around and adjusting the excited energy level. Emission occurs as photons release energy as fluorescent light¹⁸.

The extent of this effect relies on the polarity of solvent molecules where strongly polar solvent lands at a lower energy level compared to weakly polar solvent. With increasing solvent polarity, emission occurs at a lower energy level, in other words, at a more redshifted colour. Since water is slightly more polar

than ethanol, it makes sense to see dyes **1'** and **2'** slightly more bathochromically shifted in both absorbance and emission by approximately 20 nm. Not to mention, polar fluorophores display stronger sensitivity to solvent polarity. Hence, the tetra-substituted dyes (red) are undoubtedly more reactive to this phenomenon than the di-substituted dyes (orange).

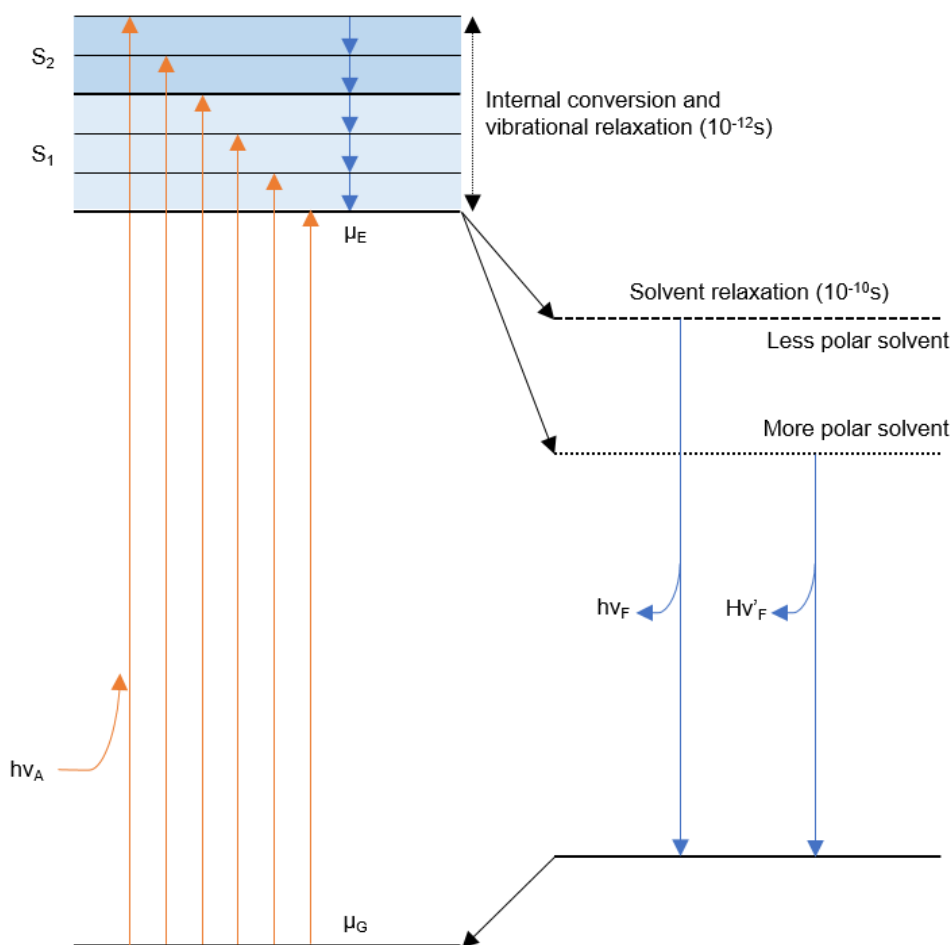


Figure 10 Jablonski diagram for fluorescence with solvent relaxation

An important criterion in determining the performance of dyes as light-converting agents is quantum yield. It measures the amount of photons emitted with respect to the amount absorbed. In this case, quantum yield represents green to orange or red conversion ratio of the dyes. Conversion efficiencies were measured using FP-8500ST spectrofluorometre at UNIST.

Dyes **1** and **2** dissolved in ethanol demonstrated fairly high quantum yield at 66% and 69%, respectively. Distinct orange and red colours were apparent from these dyes. Quantum yield of dyes **1'** and **2'** reached 29% and 35%. Florescence were not as strong, however, glowing colours were equally apparent to human eyes.

A reduction in quantum yield of dyes **1'** and **2'** in polar solvent attributes to the presence of photo-induced electron-transfer (PET) processes. The process causes a considerable broadening of the emission spectra and a low quantum yield. Since PET process heavily depends on the distance between hydrophilic substituent and chromophore, the distance between sulfonyl groups and perylene body should have led to significant PET activity and therefore resulting in lower quantum yields¹⁹. Regardless, the emission wavelengths seem to cast stronger impact on the growth rate of microalgae compared to quantum yield as the results showed better performance under bathochromically shifted light rather than under higher quantum yield dyes.

Dyes **1** and **2** and dyes **1'** and **2'** differ in structure at para-position of bay substituents. Highly polar and bulky sulfonyl groups attached in these positions

induce disproportionate electron distribution, and the ionic nature of the functional group significantly increased solubility of dyes **1'** and **2'** in water. Electron-withdrawing sulfonyl groups further extend π -conjugation from the perylene body and shift emission spectra towards longer wavelengths. The four dyes were then applied in two cultivation methodologies of either light-converting layer or light-converting medium, depending on the solubility of dyes in either ethanol or water.

As explained in the previous section, each cultivation methodologies were carried out using a pair of dyes with same substituents either di-substituted or tetra-substituted at bay positions. Since red dyes (**2** and **2'**) are more bathochromically shifted compared to the orange dyes (**1** and **1'**) in each cases, faster growth in the former samples were expected.

Part 2: Cultivation

Methodology 1: Light-converting layer

Dyes **1** and **2** dissolved at 10^{-6} M in ethanol were poured into stackable culture flasks, so-called the light-converting layers, and were placed adjacent to each cell flasks during cultivation. Lag phase lasted approximately 24 hours and continued onto exponential phase until stationary phase was reached at 96 hours.

Table 2 Lipid and biomass productivities of *Chlorella vulgaris* cultured with light-converting layer shown in percentage increases

Sample	Lipid content (%)	Lipid productivity (g / L / day)	% increase (compared to control)	Cell count (cells / mL)	% increase (compared to control)
1	11.09	0.11105	6.07	$2.080 \cdot 10^8$	31.65
2	11.74	0.11779	13.00	$1.740 \cdot 10^8$	10.83
Control	10.00	0.09996	0.00	$1.245 \cdot 10^8$	0.00

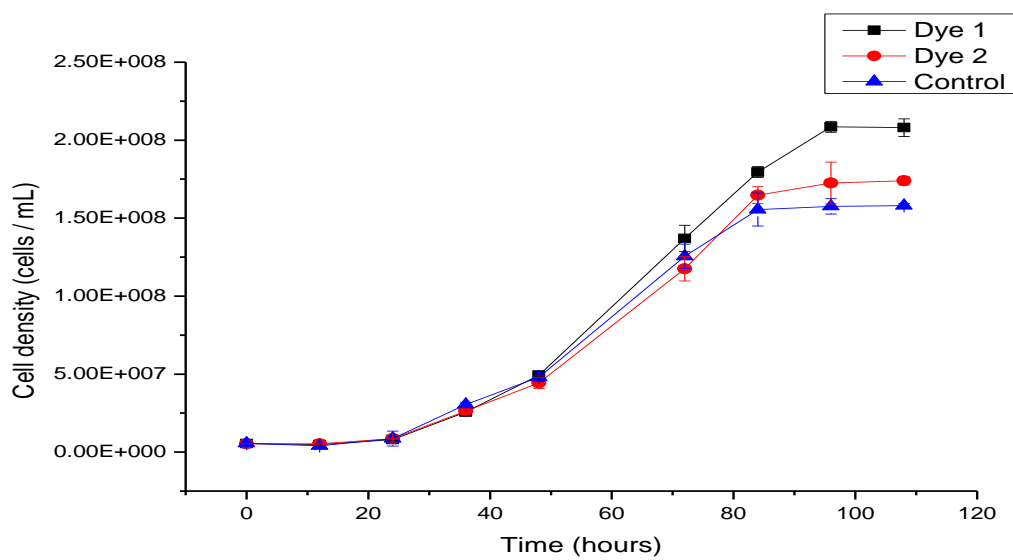
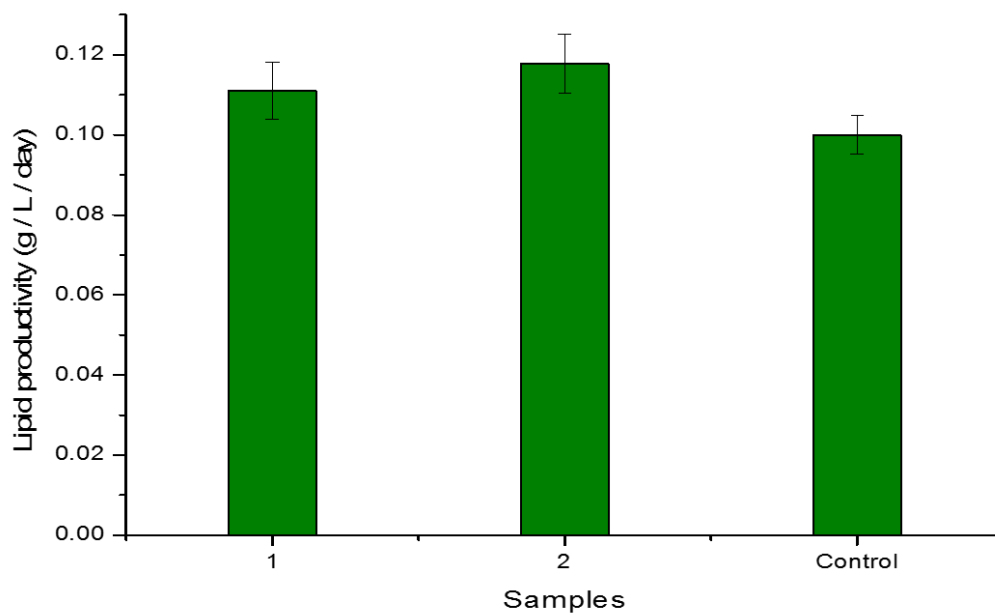


Figure 11 Lipid productivity, and cell density of *Chlorella vulgaris* cultured with light-converting layer

As hypothesized, lipid productivity increased in the order of red dye **2**, orange dye **1**, then control. Red and orange dye samples each showed lipid productivity of 0.11105 g/L/day and 0.11779 g/L/day which were equivalent to 11.10% and 17.83% escalation compared to the control at 0.09996 g/L/day.

Cell density also showed improved but in a contrasting trend to lipid productivity where the orange dye stimulated cells to replicate faster than the red dye. Dyes **1** and **2** samples reached a cell density plateau at 2.080×10^8 cells/mL and 1.740×10^8 cells/mL, respectively. These represent 31.65% and 10.83% increase compared to the control at 1.245×10^8 cells/mL. Optical density (OD) also showed the same trend to support the cell count data.

Methodology 2: Light-converting medium

For this part, preliminary tests were carried out to ensure microalgae cells can endure dye-medium environment and reproduce. Cell growth was measured by microscopic cell count and dry cell weight (DCW) after 120 hours. Cell count showed largest population size of 2.32×10^7 cells/mL in 4×10^{-5} wt% sample, and DCW weighed most heavily at 0.82 g/L in 4×10^{-6} wt% sample. This test proved that cells not only can survive in the medium, but can grow faster in it. Hence, this toxicity test turned out safe and acceptable to employ dyes in multi-functional medium, co-acting as a production medium for cells as well as a light-converting layer.

Dyes **1'** and **2'** along with cells were dissolved in the medium during cultivation and as a whole functioned as a light-converting layer. Lag phase lasted just as long as for light-converting layers. Though, the cells reached the stationary phase about half a day earlier, meaning the cell number expanded at a slightly faster rate.

Table 3 Lipid and biomass productivities of *Chlorella vulgaris* cultured with light-converting medium shown in percentage increases

Sample	Lipid content (%)	Lipid productivity (g / L / day)	% increase (compared to control)	Cell count (cells / mL)	% increase (compared to control)
1'	10.93	0.10936	4.91	$2.175 \cdot 10^8$	37.66
2'	12.20	0.12212	17.15	$1.850 \cdot 10^8$	17.09
Control	10.40	0.10424	0.00	$1.580 \cdot 10^8$	0.00

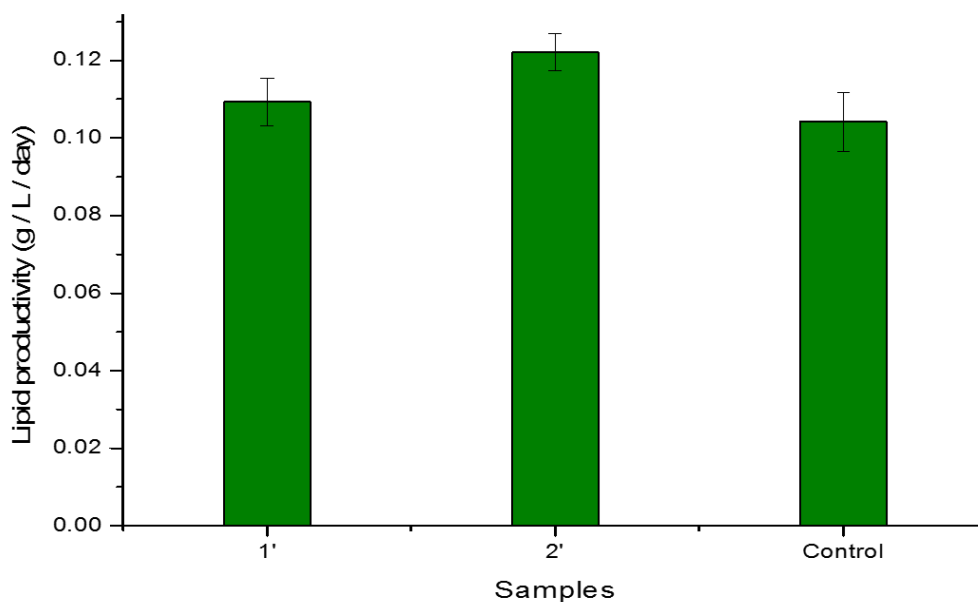


Figure 12 Lipid productivity of *Chlorella vulgaris* cultured in dye-medium

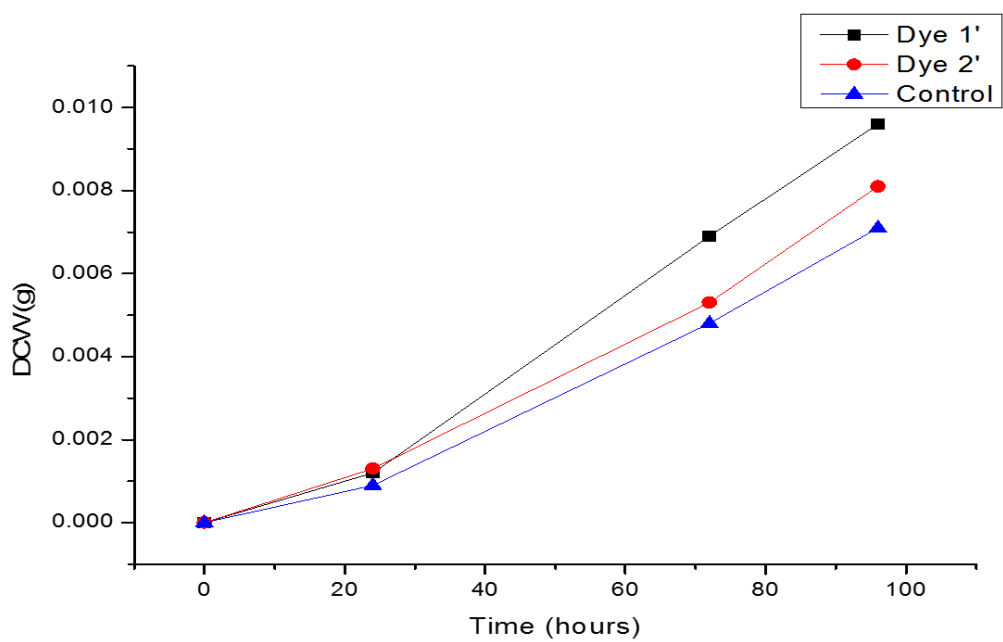
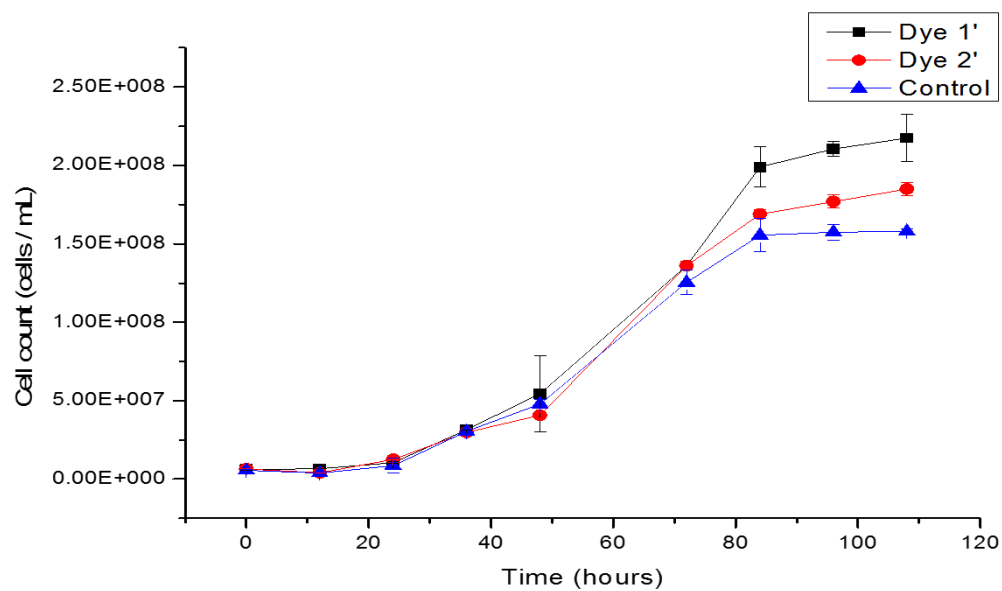


Figure 13 Cell density and dry cell weight of *Chlorella vulgaris* cultured in dye-medium

Similar to 1st batch of cultivation, the results showed resemblance in rising trends of lipid content and cell density. Lipid productivity increased in the order of red dye 2', orange dye 1', followed by control. Dyes 1' and 2' samples showed lipid productivity of 0.10936 g/L/day and 0.12212g/L/day which were equivalent to 4.91% and 17.15% rise compared to the control at 0.10424 g/L/day.

Biomass productivity increased in a reverse order where orange dye sample overtook the red dye sample, and control came last. Cell density curve reached a plateau at 2.175×10^8 cells/mL and 1.850×10^8 cells/mL, representing 37.66% and 17.09% increase from the control at 1.580×10^8 cells/mL. Also, DCW were 0.0096 g, 0.0081g, and 0.0071 g in increasing order of orange dye, red dye samples then control, once again to approve the cell density data. DCW was measured instead of OD in this case because the aqueous media were coloured by the dyes.

Data obtained showed that the use of light-converting medium was as effective as using light-converting layers. In fact, maximum lipid and biomass productivities out of all dye sample were observed in this culture batch. In comparison to light-converting layer method, less portion of incident light was lost by surface reflection, light absorption by host material, light emission from the layer edges. Given that the working volume is recycled for several cultivation cycles, light-converting medium method suggests a possibility in constructing a more space efficient, cost effective, and eco-friendly bioreactor.

Note that all quantum yield of microalgae measured at the end of cultivation cycles exceeded 70%. Quantum yield of cells measure the ratio of light absorbed by the cells to light emitted so the results can be interpreted as efficient utilization of light. It is also acceptable to correlate absorption efficiency to the rate of photosynthesis.

Table 4 Quantum yield of microalgae cells after 120-hour cultivation

Sample	Quantum yield
1	0.74
2	0.77
1'	0.76
2'	0.71
Control	0.74

Supplementary tests using increasing concentration of dyes would have strengthened data set as the results can pinpoint optimal amount of dye needed to maximize growth rate of microalgae. It is also advisable to find out exactly from what concentration the dyes begin to impede beneficial effect on the culture cells, predominantly caused by screening.

Similar trials on different light intensities controlled by light power, or varying colours such as blue, yellow, or green, are also recommended for future study.

CHAPTER V: CONCLUSION

It is logical to conclude that spectral conversion can improve lipid and biomass productivities of *Chlorella vulgaris*. Four fluorescent dyes absorbed mostly in green wavelength range and emitted in orange and red regions to allow more light energy available for photosynthesis. Light alteration also provides environmentally friendly, economically feasible cultivation technique for prosperous production of lipid or biomass, ultimately for efficient biofuel production.

Cultivation results showed that bathochromically-shifted dyes improved lipid productivity more extensively. Specifically, red dye samples increased in lipid productivity more than orange dyes samples in both light-converting layers and light-converting media. Orange dye samples instead outperformed in biomass productivity where population size expanded more in orange dye samples more than red samples. The effect of light on lipid and biomass productivities are colour specific.

Light-converting layer and light-converting medium methodologies both promoted growth rate of *C. vulgaris*. The implications of the second method are significant in the area of bioreactor design as they overcome many critical limitations found in conventional layered structures built for spectral conversion.

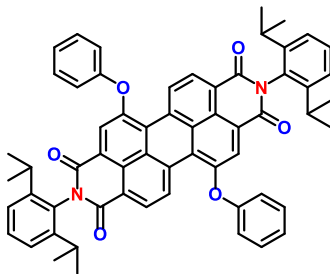
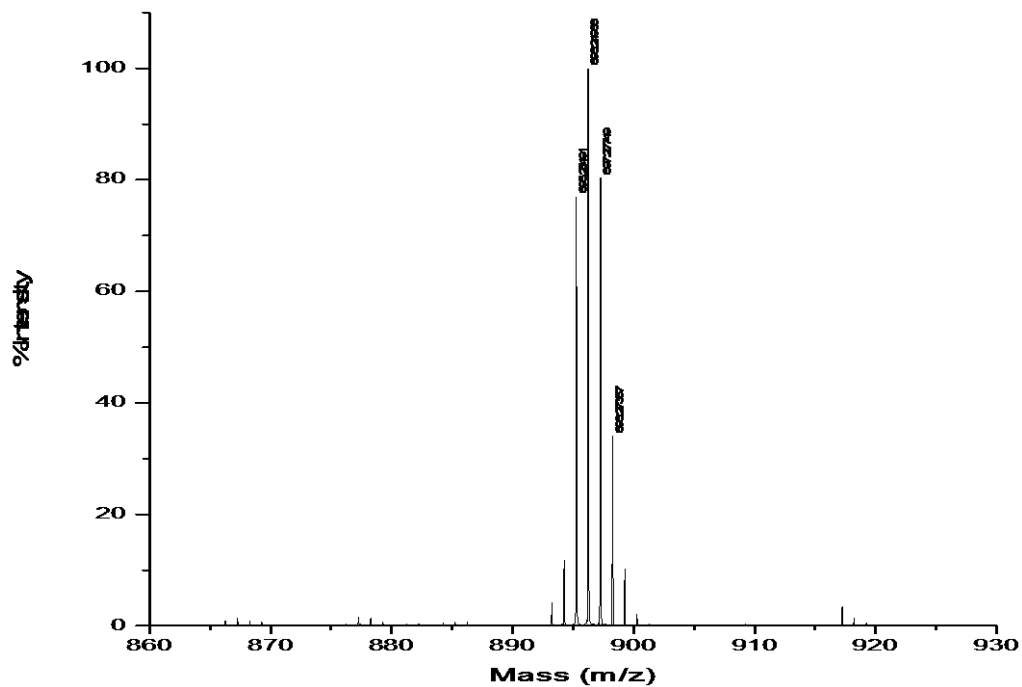
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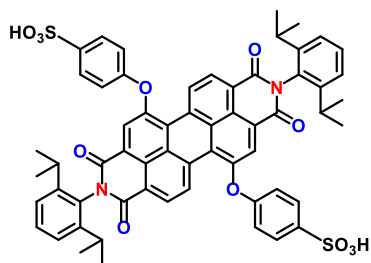
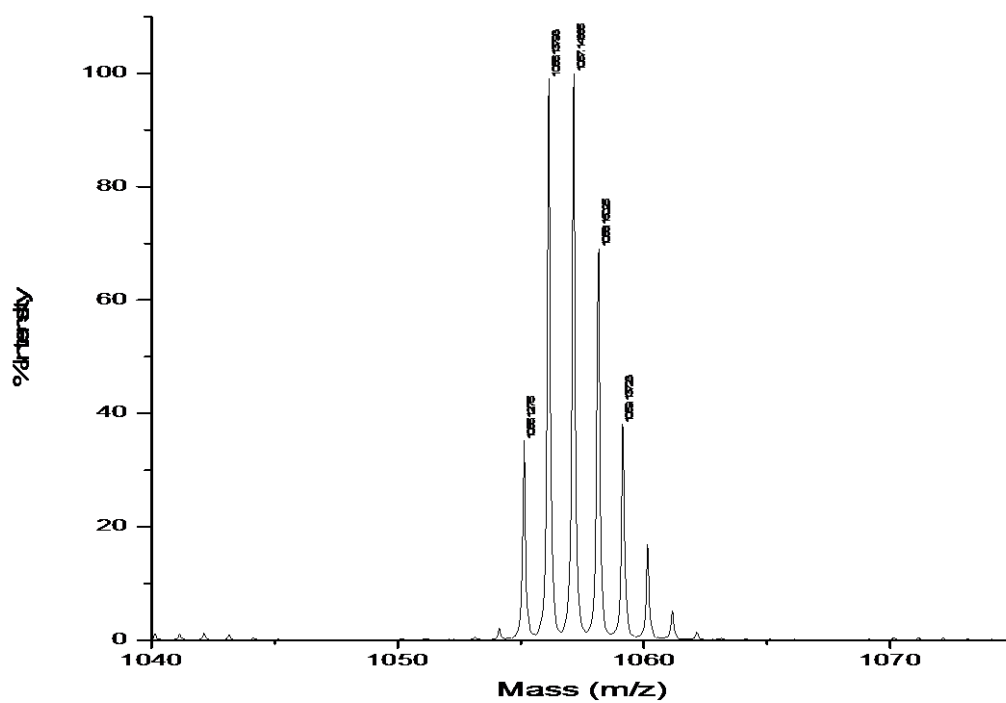
Appendix: Matrix-Assisted Laser Desorption Ionization – Time of Flight

Dye 1



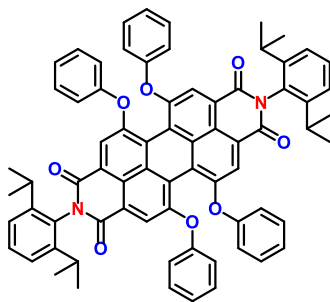
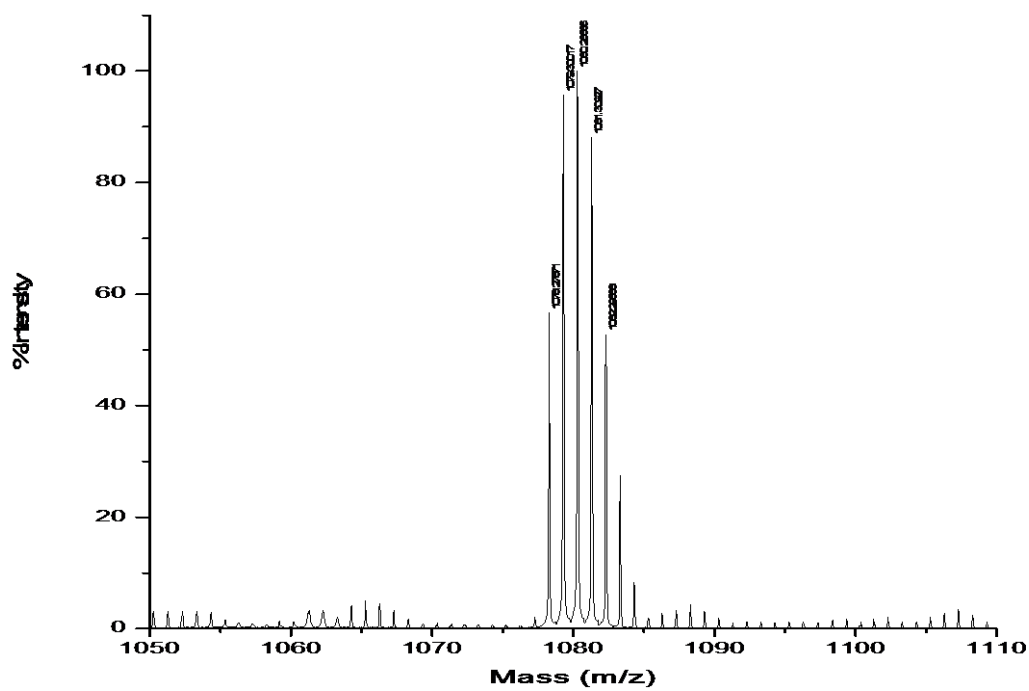
MALDI-TOF MS: m/z 896.25, 100% [M + H⁺]

Dye 2



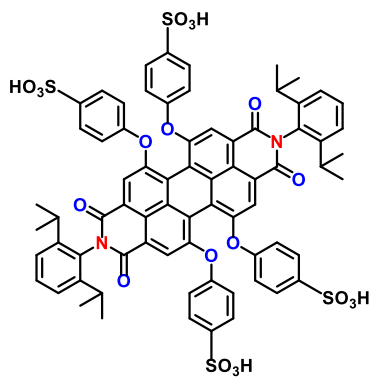
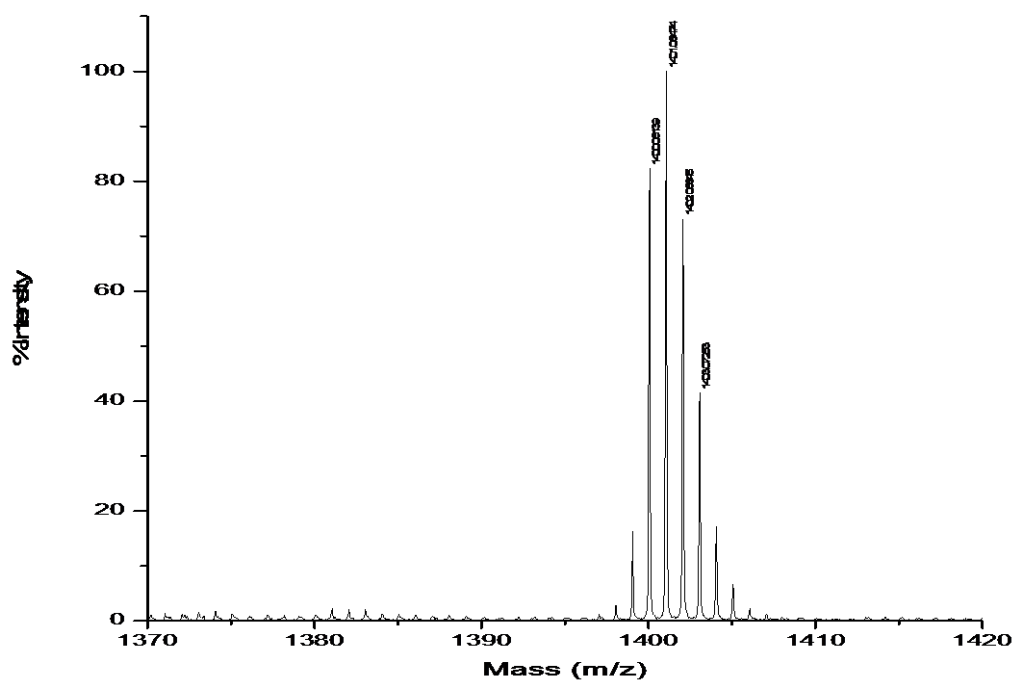
MALDI-TOF MS: m/z 1057.15, 100% [M + H⁺]

Dye 1'



MALDI-TOF MS: m/z 1080.29, 100% $[M + H^+]$

Dye 2'



MALDI-TOF MS: m/z 1401.06, 100% [M + H⁺]

Summary

본 연구에서 광전환을 일으키기 위해 합성된 적색 형광 염료를 미세조류 배양 시스템에 적용하여 하나인 *Chlorella vulgaris* 의 성장률을 향상시키는 것을 확인하였다. 이는 광전환 형광 염료가 미세조류의 성장에 필수적인 파장대의 빛을 추가적으로 제공함으로써 세포 내 lipid 의 생산성을 높이는 것에 집중하여 수행되었다.

Perylene 을 모체로 한 4 종의 형광 염료는 녹색 파장대(500 – 580 nm)에서 가장 효율적으로 흡광하고 적색 파장대(580 – 650 nm)에서 발광하도록 설계하였다. 그 중 비수용성 염료 2 종은 유기용매에 녹여 광원과 미세조류 배양액 사이에 광전환 층을 생성하는 방법으로, 수용성 염료 2 종은 배지에 미세조류와 함께 섞는 방법으로, 미세조류의 배양 시스템에 적용되었다.

두 가지 방법으로 배양한 결과, 더 장파장화된 빛을 발산하는 염료 2 와 2' 를 적용한 경우에서 *C. vulgaris* 가 대조군에 비해 각각 최대 13.00% 와 17.15%의 높은 지질 생산성을 보였으며, 염료 1 과 1' 를 적용한 경우에는 바이오매스 성장률이 대조군 대비 각각 최대 31.65%와 37.66%로 증가하였다.

이러한 결과를 통해 적색 형광 염료의 광전환이 미세조류의 성장을 증가시킨다는 것을 입증하였다. 또한 이와 같은 배양법은 미세조류의 생산성을 증가시켜 대체에너지 자원으로서 미세조류의 활용도를 크게 증대시킬 것으로 기대된다.